Journal of Cellular Biochemistry 101:68–79 (2007)

Bystander Killing of Breast Cancer MCF-7 Cells by MDA-MB-231 Cells Exposed to 5-Fluorouracil Is Mediated via Fas

Rishi Raj Chhipa and Manoj Kumar Bhat*

National Centre for Cell Science, Ganeshkhind, Pune 411 007, India

Abstract The major drawback with cancer therapy is the development of resistant cells within tumors due to their heterogeneous nature and due to inadequate drug delivery during chemotherapy. Therefore, the propagation of injury (''bystander effect'' (BE)) from directly damaged cells to other cells may have great implications in cancer chemotherapy. The general advantage of the bystander cell killing phenomenon is the large therapeutic index that can be achieved. Experiments suggest that this phenomenon is detected in radiation therapy as well as in gene therapy in conjunction with chemotherapy. In the present study, we developed an original in vitro model dedicated to the exploration of bystander cytotoxicity induced during breast carcinoma chemotherapy. In brief, we investigated this perpetuation of injury on untreated bystander MCF-7 breast cancer cells which were coplated with 5-fluorouracil (5-FU)-treated MDA-MB-231 breast cancer cells. To achieve this goal, a specific in vitro coculture model which involved mixing of aggressive MDA-MB-231 breast cancer cells with enhanced green fluorescent protein (EGFP) expressing stable clone of non-metastatic MCF-7 breast cancer cells (MCF-EGFP), was used. A bystander killing effect was observed in MCF-EGFP cells cocultured with MDA-MB-231 cells pretreated with 5-FU. The striking decrease in MCF-EGFP cells, as detected by assaying for total GFP intensity, is mediated by activation of Fas/FasL system. The implication of Fas in MCF-EGFP cell death was confirmed by using antagonistic anti-FasL antibody that reverses bystander cell death by blocking FasL on MDA-MB-231 cells. In addition, inhibition of CD95/Fas receptor on the cell surface of MCF-EGFP cells by treatment with Pifithrin-alpha, a p53 specific transactivation inhibitor, partially abrogated the sensitivity of bystander MCF-EGFP cells. Our data, therefore, demonstrates that the Fas/FasL system could be considered as a new determinant for chemotherapy-induced bystander cell death in breast cancers. J. Cell. Biochem. 101: 68–79, 2007.

Key words: bystander effect; p53; Fas; 5-FU; breast cancer

Solid tumors are difficult to treat, though surgery is performed where it is possible, chemotherapy is mostly the choice of treatment. One of the major limitations of chemotherapy is the inability of anticancer agents to induce cytotoxicity due to inaccessibility to certain cells in a tumor. Moreover, histopathological heterogeneity in human tumors is a well-documented phenomenon. It is now widely admitted that breast carcinoma is a genetically and clinically heterogeneous disease as it contains different clones of tumor cells arising from continual differentiation of transformed progenitor cells [Symmans et al., 1995; Konemann et al., 2000; Sharifi-Salamatian et al., 2004; Li and Rosen, 2005]. Since cancer now is considered more as a deficiency of apoptosis rather than a mere proliferation issue [Guchelaar et al., 1997], additional mechanisms which induce or enhance cell death during cancer chemotherapy will be beneficial for the better outcome of the treatment. Though determining the factors involved in drug response is a new challenge in modern cancer chemotherapy [Van Triest et al., 2000], a new phenomenon called "The Bystander Effect" (BE) has been attributed in several investigations for the propagation of injury in cancer therapy. The name was initially borrowed from the gene therapy field, where it

Grant sponsor: Department of Biotechnology, Government of India.

*Correspondence to: Dr. Manoj Kumar Bhat, Scientist E, National Centre for Cell Science, NCCS Complex, Ganeshkhind, Pune 411 007, India.
E-mail: manojkbhat@nccs.res.in, manojkbhat@yahoo.com

Received 29 June 2006; Accepted 29 August 2006
DOI 10.1002/jcb.21153

usually referred to the killing of several types of tumor cells by targeting only one type of cell within a mixed population [Cheng et al., 1999].

The BE is a chemo and radiobiological phenomenon that has come to the force recently. It describes the ability of cells affected by an agent to convey manifestations of damage to other cells neither directly targeted by the agent nor necessarily susceptible to it per se. Thus, the BE is elicited indirectly by communication between cells which are directly affected by agent and those that are not. While the term BE may cover a variety of distinct mechanisms, the common denominator is cellular interaction, either to increase or decrease viability of non-target cells [Djordjevic, 2000]. The data available concerning the BE falls into two quite separate categories. First, there are experiments involving the transfer of medium from treated cells (effector cells), to untreated cells (target cells) resulting in biological effect on later cells. Second, there is the use of specifically treated effector cell population and BEs are studied in the target cells cocultured subsequently with these effector cells. Several reports have described utilization of mixed population of cells and cocultures to study the BEs [Kagawa et al., 2001; Shao et al., 2005]. These cocultures were constituted by mixing either homogenous or heterogeneous population of cells [Hofmann and Blau, 1997; Tanaka et al., 2001]. BE has been shown for cell lethality, chromosomal aberrations, and cell cycle delay [Scott, 2004]. The type of cells involved and type of treatment appear to be an important determinant. Therefore, the bystander studies suggest that biological effects of any anticancer therapy are larger than the assumed outcome of these treatments.

The bystander cytotoxicity using gene therapy in conjunction with chemotherapy for example, Herpes Simplex Virus Thymidine kinase gene utilized for activation of prodrug Ganciclovir, has been described in several investigative reports [Grignet-Debrus et al., 2000; Mesnil and Yamasaki, 2000]. Additionally, the role of factors like p53, *TRAIL* gene, and Cytochrome P-450 in bystander cell death in cancer cells has been reported by some laboratories [Xu et al., 1997; Zhou et al., 2000; Kagawa et al., 2001]. Moreover, this effect has also been demonstrated in cancer cells exposed to both high- and low-energy radiations in several separate experimental models [Albanese and Dainiak, 2000; Hall, 2003; Mothersill and Seymour, 2004]. It has been demonstrated that following a low dose of alpha particles, a larger population of cells manifested biological damage than those actually have been hit by alpha particles [Nagasawa and Little, 1992; Hall, 2003]. Chemotherapeutic drugs can induce apoptosis and upregulate death ligands or their receptors which may subsequently play a significant role in death signal amplification via BE in a mixed population of cells. Significant number of investigations have shown apoptosis mediated via Fas/FasL system in cancer cells treated with anticancer agents [Friesen et al., 1999; Mo and Beck, 1999]. However, the implication of chemotherapeutic stress-induced bystander cell death has been least investigated regimen in cancer biology.

In the present study, we developed clones from breast cancer cells, MCF-7 and MDA-MB-231, (MCF-EGFP and MDA-MB-231-EGFP respectively), which stably expressed enhanced green fluorescent protein. We utilized them as an expeditious tool to study bystander cell death phenomenon in a mixed culture assays. We investigated chemotherapeutic agent 5-fluorouracil (5-FU) or Carboplatin (Carb) induced cytotoxicity either in MDA-MB-231 or MCF-7 cells (drug treated cells were termed as "effector cells") on bystander MCF-EGFP or MDA-MB-231-EGFP cells (EGFP expressing cells which were not treated with drugs were termed as "target cells"). The BE-mediated toxicity on target cells was evaluated by measuring the green fluorescence intensities. We, for the first time, demonstrated that MCF-EGFP target cells die when cocultured with MDA-MB-231 effector cells that were pretreated with 5-FU. This phenomenon is both drug and cell type specific and is dependent on membrane bound death receptor/death ligand, Fas/FasL system. To our knowledge, no comparable systematic study on the efficacy of chemotherapy-induced bystander killing targeted towards cancer treatment exists.

MATERIALS AND METHODS

Reagents and Antibodies

Sources of materials were as follows: 5-fluorouracil (5-FU), Carboplatin (Carb), 8-Bromo-cyclic-AMP (BCMP), and Pifithrin alpha (PFTα) were purchased from Sigma, MO. Carb and 5-FU were dissolved in sterile water to prepare 50 mM stock. PFTα was dissolved in

DMSO. BCMP was dissolved in sterile water to prepare 1 M stock solution. Antagonistic anti-FasL antibody and anti TRAIL antibodies (BD Biosciences, CA) were reconstituted in sterile PBS as 1 mg/ml stocks.

Cell Cultures and Development of MCF-EGFP and MDA-MB-231-EGFP Cell Lines

Human breast cancer lines MCF-7 (ATCC HTB-22) and MDA-MB-231 (ATCC HTB-26) were obtained from ATCC (Manassas, VA) and maintained in our in-house National Cell repository. Cells were routinely cultured in DMEM and F12K (1:1) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, UH), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, CA), at 37°C with 5% CO_2. The MCF-EGFP and MDA-MB-231-EGFP cell lines, expressing green fluorescent protein were established as follows. The MCF-7 and MDA-MB-231 cells were separately transfected with pEGFPN1 plasmid (Clontech, CA) by calcium phosphate method and selected on G418 (USB, OH) 800 and 600 μg/ml, respectively. Subsequently, the cells were maintained in medium containing G418 (100 μg/ml).

Bystander Cytotoxicity

We designed an in vitro coculture experiment to evaluate whether the cytotoxicity induced by commonly used chemotherapeutic agents such as 5-FU or Carb, either in MDA-MB-231 or MCF-7 breast cancer cells (drug treated cells were termed as effector cells) leads to death of coplated bystander MCF-EGFP or MDA-MB-231-EGFP cells (EGFP expressing breast cancer cells which were not treated with drugs were termed as target cells). The bystander EGFP positive target cells were evaluated for cell death by quantification of total live fluorescence by microfluorimetry with an absorbance at 488 nm and emission at 510 nm (Fluoroskan Ascent FL, Labsystems, Finland). The fluorescence intensities of bystander target cells coplated with untreated effector cells were plotted as 100%. Briefly, the exponentially growing effector cells (50,000 per well) were plated in a 24-well culture plate and after allowing them to adhere for 24 h, cells were treated with 100 or 500 μM of 5-FU or Carb for additional 24 h. Subsequently, effector cells were washed twice with medium and the target cells were coplated (50,000 per well) without or with PFTα (20 μM), DMSO, FasL or TRAIL antagonistic antibodies (500 ng/ml each), or BCMP (1 mM) treatments, as required for the experiments. After 48 h of growth in coculture, both target and effector cells were trypsinized and quantitated for EGFP intensity of surviving fraction of target cells, as described above. In the investigations where conditioned media from effector cells was assayed for its toxicity on bystander target cells, the effector cells were pretreated with the anticancer agent for 24 h. After washing twice, the effector cells were incubated further in complete medium for 48 h and subsequently, this medium was used for culturing target EGFP positive cells for 48 h before reading fluorescence.

Subcellular Fractionation

The cytosolic and membrane fractions were obtained by ultracentrifugation [Nakajima et al., 2000]. Briefly, cells were removed from plates using rubber policeman and homogenized in ice cold buffer containing 20 mM Tris-HCl, pH 7.6, 10 mM EDTA, 10 mM EGTA, 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 100 mM NaF, 10 mM sodium pyrophosphate, 2 mM Na_3VO_4, 1 mM PMSF, 10 μM leupeptin, 10 μg/ml aprotinin, and 1.5 μM pepstatin. Then homogenate was centrifuged at 100,000*g* for 60 min at 4°C and supernatant was collected as cytosolic fraction. The pellet was washed with the same buffer, resuspended in buffer containing 1% Triton X-100, and recentrifuged at 100,000*g* for 60 min at 4°C. The resultant supernatant was collected as the membrane fraction. The fractions thus obtained were immunoblotted as described below.

Western Blot Analysis

Following treatments, cells were washed thrice with ice cold PBS and lysed in 100 μl of ice-cold lysis buffer (20 mM HEPES pH 7.4 containing 1% NP-40, 2 mM EGTA, 2 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 mM TPCK, 2 mM Na_3VO_4, and protease inhibitor cocktail tablet) per 1×10^6 cells. Samples were boiled in SDS sample buffer for 10 min followed by separation on an SDS–PAGE. Equal amount of protein samples (75 μg) were resolved on 10–12% SDS–polyacrylamide gel and then transferred onto nitrocellulose membranes (Amersham, Aylesbury, UK). The membranes were probed with antibodies against p53, estrogen receptor-

alpha, Fas ligand caspase-8, and β-actin (Santacruz Biotechnology, CA). The immunoblots were detected by enhanced chemiluminescence (ECL) reagent (New England Biolabs, MA).

Reverse Transcription Polymerase Chain Reaction Analysis for FasL Expression in MDA-MB-231

Total cellular RNA from treated and untreated cells was extracted using TRIzol™ reagent (Invitrogen Life Technologies, CA), according to the manufacturer's instructions. Five micrograms of total RNA and oligo $(dT)_{12-18}$ primer was taken in diethyl pyrocarbonate (DEPC)-treated water. cDNA synthesis was initiated using 200 U of M-MLV reverse transcriptase (Invitrogen Life Technologies), under conditions recommended by the manufacturer and the reaction was allowed to proceed at 37°C for 50 min. Reaction was terminated by heating at 70°C for 15 min. Each RT-PCR contained 10% of cDNA, 20 pM of each primer in 20 mM Tris-HCl (pH 8.4) containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, and 1 U of platinum *Taq* DNA polymerase (Invitrogen Life Technologies) in a final volume of 20 μl. After an initial denaturation for 2 min at 95°C, 30 cycles of denaturation (94°C for 1 min), annealing (for 1 min), and extension (72°C for 1 min) were performed on a DNA thermal cycler (Techne, Cambridge, UK) with a final extension for 10 min at 72°C. The primer pairs used were as follows: FasL 5′-GTC AAT CTT GCA ACA ACC TGC-3′ (F) 5′-ACA ACA TTC TCG GTG CCT G-3′ (R) and β-actin 5′-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3′ (F) 5′-CGT CAT ACT CCC TGC TTG CTG ATC CAC ATC TGC-3′ (R). The annealing temperatures for FasL and β-actin PCR were 57 and 55°C, respectively.

Apoptotic Death Detection by Annexin V-PE Staining

To evaluate whether the death of target cells (MCF-EGFP) is due to induction of apoptosis, we carried out annexin V-PE staining and cells were analyzed by flowcytometry. MCF-EGFP cells were cultured alone, cocultured with untreated effector MDA-MB-231 cells or cocultured with drug-treated effector MDA-MB-231 cells according to requirement of the experiment, as described in the methodology earlier. Briefly, cells were washed with PBS and stained with annexin V-PE according to the manufacturer's protocol (Apoalert Annexin V-PE Apoptosis Kit; Clontech, CA). Washed cells were subjected to FACScan flow cytometer (Becton Dickinson Gmbh, Heidelberg, Germany). Forward scatter (FSC) and orthogonal scatter (SSC) were collected using linear amplification. Annexin V-PE and EGFP fluorescence was collected using log amplification and 10,000 events were recorded. Cells were analyzed using quadrant statistics in an annexin V-PE versus EGFP dual parameter histogram. Cell populations were expressed as percentages.

Statistical Analysis

Data are expressed as the mean of three independent results. Statistical comparisons are made using Student's *t*-test and *P*-value <0.05 was considered as significant.

RESULTS

Chemotherapy Induced Bystander Cytotoxicity in the Mixed Cultures of Breast Carcinoma Cells

Bystander cytotoxicity has been projected essentially as a radiobiological as well as gene therapy associated phenomenon in cancer biology, thus, our quest was to investigate whether it also occurs during cancer chemotherapy. We developed stable cell lines which constitutively express EGFP. The MCF-EGFP and MDA-MB-231-EGFP cells lines did neither exhibit any significant alterations in their growth properties nor do they exhibit any alterations in sensitivity to chemotherapeutic drugs as compared to parental cells (data not shown). We established an appropriate model by treating MDA-MB-231 or MCF-7 breast cancer cells with 5-FU or Carb and subsequently coplating either MCF-EGFP or MDA-MB-231-EGFP cells. Results thus obtained demonstrated that 5-FU-treated MDA-MB-231 induced bystander killing in MCF-7 cells as detected by measuring total GFP intensity (Fig. 1B) or by observing cells under a fluorescent microscope (Fig. 2, panel e). By quantification of green fluorescence, it was also established that MCF-7 cells treated with 5-FU or Carb at any of the two doses (100 and 500 μM), did not induce bystander killing in either MCF-EGFP (Fig. 1A) or in MDA-MB-231-EGFP cells (Fig. 1C). This observation was supported by fluorescent photomicrographs of target bystander MCF-EGFP (Fig. 2, panels b and c) or in target bystander MDA-MB-231-EGFP cells (Fig. 2, panels h and i). The fluorescent photomicrographs are representative of observations where 500 μM of drugs

were used. Similarly, 5-FU and Carb-treated MDA-MB-231 did not induce bystander killing in MDA-MB-231-EGFP cells (Fig. 1D). The representative photographs (Fig. 2, panels k and l) from the coculture were in support of fluorescence intensity data. However, MDA-MB-231 cells treated with varying doses of 5-FU induced bystander killing of MCF-EGFP cells in a dose dependent manner ($^*P < 0.05$ vs. untreated effector cells). 5-FU at a dose of 100 and 500 μM led to decrease in GFP intensity by 15 and 40%, respectively (Fig. 1B). In contrast, no such bystander killing of MCF-EFGP cells was detected with Carb treated MDA-MB-231 cells (Figs. 1B and 2, panel f). Data presented here clearly demonstrates that 5-FU-treated effector MDA-MB-231 cells induce bystander killing of target MCF-EGFP cells.

The Bystander Cytotoxicity in Target MCF-7 Cells Is Mediated Via Membrane Bound Fas Ligand Expressed on the Effector Cells

Since 5-FU-treated MDA-MB-231 induced bystander killing in MCF-7 cells, we next explored the molecular alterations that may be involved in the cell killing. We investigated the involvement of death ligands like, Fas ligand (FasL) and TRAIL. In several studies, both these ligands have been reported to be involved in bystander cell death [Kagawa et al., 2001; Ciccolini et al., 2002; Hyer et al., 2003]. To investigate the involvement of death ligands in bystander killing of MCF-7 cells by 5-FU (500 μM)-treated MDA-MB-231 cells, we performed coculture experiments in the presence of FasL and TRAIL antagonistic antibodies as described in the materials and methods. In brief, the effector cells were 5-FU treated, washed, and the target cells were plated in media in the presence of antagonistic antibodies for 48 h. As shown in Figure 3A, when the bystander EGFP positive cells were coplated with the treated effector cells in presence of antagonistic FasL antibody, the EGFP intensity of target cells was similar to that of cells plated with control cells, suggesting involvement of FasL in the bystander cell killing ($^*P < 0.05$ vs. drug-treated effector cells). Under similar experimental

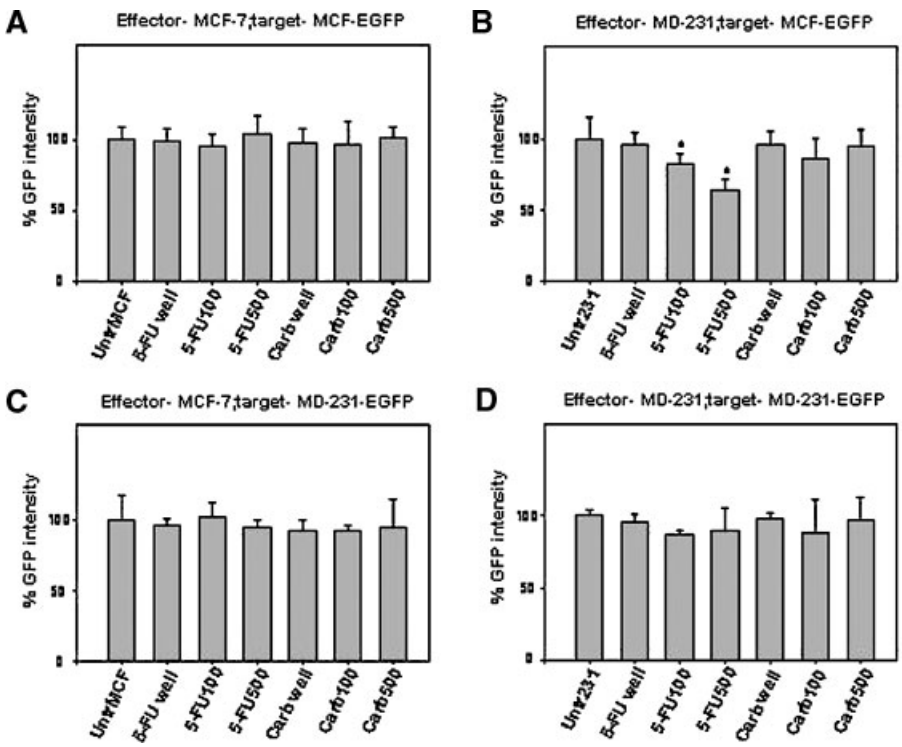

Fig. 1. Coculture experiments to evaluate chemotherapy-induced bystander killing in breast cancer cells. **A**, **C**: Bystander killing effect mediated by effector MCF-7 cells treated with 5-FU and Carb (100 and 500 μM doses for each drug) for 24 h, towards the target MCF-EGFP and MDA-MB-231-EGFP cells, respectively. **B**, **D**: Bystander killing effect mediated by effector MDA-MB-231 cells treated with 5-FU and Carb (100 and 500 μM doses for each drug) for 24 h, towards the target MCF-EGFP and MDA-MB-231-EGFP cells, respectively. Data represent mean ± SD from three independent experiments ($^*P < 0.05$ vs. untreated effector cells).

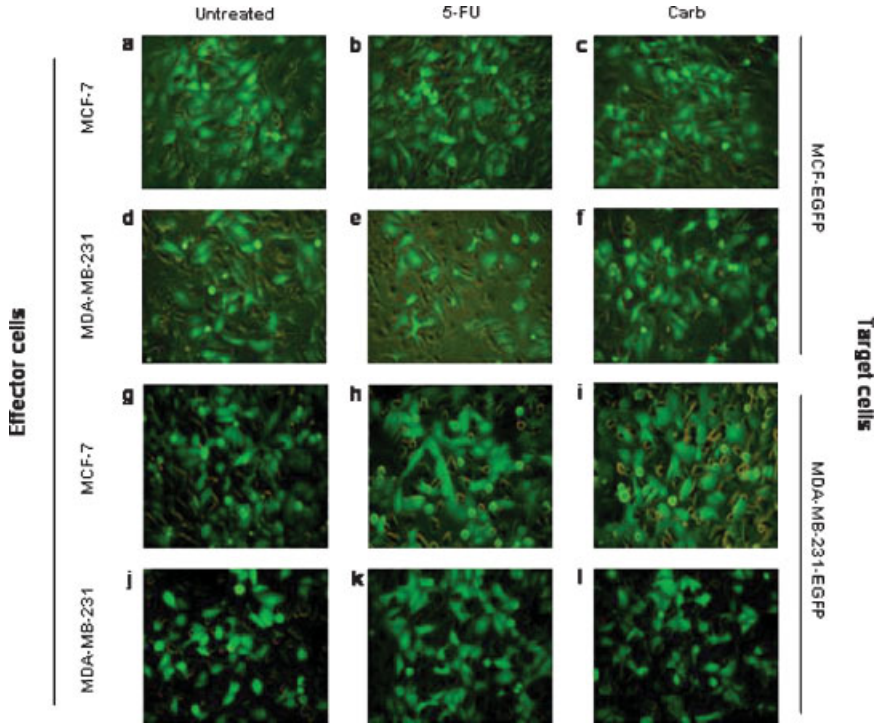

Fig. 2. Representative photomicrographs of coculture experiments to evaluate chemotherapy-induced bystander killing in breast cancer cells. Bystander killing effect on MCF-EGFP cells plated with (**a**) untreated MCF-7 cells, (**b**) 5-FU (500 μM)-treated MCF-7 cells, (**c**) Carb (500 μM)-treated MCF-7 cells, (**d**) untreated MDA-MB-231 cells, (**e**) 5-FU (500 μM)-treated MDA-MB-231 cells, (**f**) Carb (500 μM)-treated MDA-MB-231 cells. Bystander killing effect on MDA-MB-231-EGFP cells plated with (**g**) untreated MCF-7 cells, (**h**) 5-FU (500 μM)-treated MCF-7 cells, (**i**) Carb (500 μM)-treated MCF-7 cells, (**j**) untreated MDA-MB-231 cells, (**k**) 5-FU (500 μM)-treated MDA-MB-231 cells cells, (**l**) Carb (500 μM)-treated MDA-MB-231 cells. Cells were cultured as monolayer on tissue culture dish and photographed using fluorescent microscope to show cell growth in coculture. Representative fields were photographed at 20× magnifications. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

conditions when TRAIL antagonistic antibody was utilized, no consequent increase in the EGFP intensity of target cells was observed, suggesting its non-involvement. The bystander transmission of cell death signal may be either by direct interaction of membrane bound Fas ligand of effector cells and Fas receptor of target cells following cell-to-cell contact or by some soluble FasL [Schneider et al., 1998] released by stressed effector cells in the cocultures. Therefore, complementary studies were carried out which involved the transfer of medium from the treated effector cells to the target cells. As shown in Figure 3B, when the target cells were grown in the media obtained from the treated effector cells, no decrease in EGFP intensity was observed, indicative of complete absence of cytotoxic soluble factor in the culture supernatants ($^*P < 0.05$ vs. drug-treated effector cells). Moreover, separate experiments showed that GJIC (gap junction-mediated intercellular communication) was also not involved in the transmission of cytotoxicity from effector to target cells as the specific GJIC enhancer, BCMP, did not enhance the killing (Fig. 3B) of MCF-EGFP cells in coculture [Azzam et al., 2001, 2003; Chipman et al., 2003]. Taken together, these results imply that membrane bound FasL-induced death signal is indeed involved in decrease in the EGFP intensity of bystander target cells.

Membrane Bound FasL Expression Is Upregulated in 5-FU-Treated MDA-MB-231 Effector Cells

We next investigated whether FasL and its cognate Fas receptor expression is upregulated in effector MDA-MB-231 cells following drugs treatment. MDA-MB-231 cells were treated with 500 μM each of 5-FU and Carb separately, and membrane fractions were prepared and analyzed for FasL and Fas receptor expression. Interestingly, as shown in Figure 3C, in MDA-

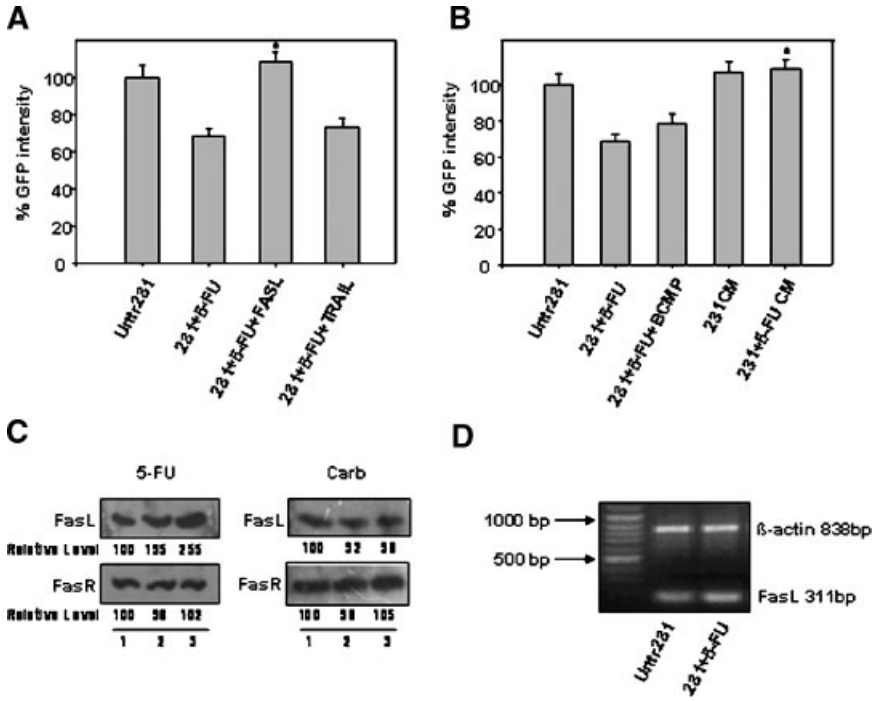

Fig. 3. Bystander cell killing is due to enhanced FasL expression on effector cells. **A**: Effect of antagonistic antibodies to FasL and TRAIL on the cell death of bystander MCF-EGFP cells (*$P < 0.05$ vs. drug-treated effector cells). **B**: Effect of conditioned media and GJIC enhancer, BCMP. GFP intensity was taken as 100% for cocultures where effector cells were not treated with 5-FU. Error bars represent the standard deviation of the mean from the results of experiments done in triplicate (*$P < 0.05$ vs. drug-treated effector cells). **C**: Western blot analysis for FasL and Fas receptor (FasR) protein expression in MDA-MB-231 cells (**lane 1**) untreated, (**lane 2**) treated with 5-FU or Carb for 24 h, and (**lane 3**) treated with 5-FU or Carb for 24 h, and further allowed to grow for 24 h in the absence of drug. Relative levels were measured by densitometry with control normalized to 100. **D**: PCR analysis of FasL mRNA expression by MDA-MB-231 cells and 5-FU-treated MDA-MB-231 cells. The positions of β-actin and FasL transcripts are shown.

MB-231 cells, treatment with 5-FU for 24 h leads to significant increase in FasL levels which was enhanced further if the cells are allowed to grow for additional 24 h in drug-free media following the treatment. Under similar experimental conditions, no increase in FasL was observed in Carb-treated MDA-MB-231 cells. Moreover, no alterations in Fas receptor were detected in MDA-MB-231 cells treated with either drug. Further, the increase in FasL at the level of transcription was confirmed by RT-PCR analysis of RNA from 5-FU (500 μM)-treated MDA-MB-231 cells (Fig. 3D). Overall, these results indicate that in MDA-MB-231 cells, 5-FU treatment not only enhanced FasL mRNA, but it also resulted in increase in its localization on the membrane thereby facilitating bystander killing of target MCF-EGFP cells.

p53 and Fas Receptor Contributes to the Bystander Killing of Target MCF-EGFP Cells

The biological effects of FasL are mediated by the expression of Fas receptor (Fas) on the cell surface and p53 plays a major role in directly regulating its expression [Bennett et al., 1998; Muller et al., 1998]. Therefore, the bystander cell killing of target cells MCF-EGFP was examined for its dependency on tumor suppressor p53. The target cells were coplated on 5-FU-treated effector cells in the absence or presence of PFTα, a potent and specific inhibitor of p53 transactivity. Interestingly, as shown in Figure 4A, in the presence of PFTα, the EGFP intensity of target cells was significantly more than either DMSO treated or experimental cells, indicating the survival of target cells (*$P < 0.05$ vs. drug-treated effector cells). Subsequently, we next examined whether inhibition of p53 activity alters the levels of Fas receptor on the membrane of the target cells. As shown in Figure 4B, treatment of cells with PFTα leads to decrease in the expression levels of Fas receptor when compared to the control cells or DMSO-treated cells. As compared to control or DMSO-treated cells, PFTα treatment causes almost 45% decrease in Fas receptor

level in target cells (Fig. 4B, lanes 1 and 2 vs. lane 3). Under the similar treatment conditions, no significant decrease in either the p53 or membrane associated estrogen receptor alpha (ERα) was detected. These results indicate that presence of Fas receptor and p53 activity is involved in mediating cell death in the target bystander cells.

Fas/FasL Activates Procaspase-8 in Target Cells

Caspases play a major role in execution of death signal induced by various apoptotic stimuli. Caspase-8 is a part of this growing family of cysteine proteases that were shown to be involved in many forms of apoptosis. Caspase-8 functions downstream of the death receptor-mediated pathways involving CD95/Fas and tumor necrosis factor alpha receptor [Ashkenazi and Dixit, 1998]. Moreover, all caspases are synthesized as inactive proenzymes that have to be activated by proteolytic cleavage. We determined the extent of conversion of procaspase-8 to active caspase-8 by immunoblot analysis. As shown in Figure 5A, significant decrease in the cellular procaspase-8 levels was observed in the lysate prepared from cocultures of 5-FU-treated effector cells and target cells when compared to the protein levels in cocultures where no 5-FU was added to effector cells (lane 5 vs. lane 3). In the coculture conditions, the effector and target cells were plated in 1:1 ratio, as described in Materials and Methods, and equal protein was resolved in each lane. The decrease in procaspase-8 has been attributed to its activation and corresponds to conversion of pro-enzyme to its active subunits following association of activated Fas and death-inducing signaling complex [Scaffidi et al., 1998]. The unchanged procaspase-8 level in lane 4 indicates that the decrease in procaspase-8 levels is not contributed by drug-treated effector cells. Moreover, no decrease in procaspase-8 levels was detected in untreated MCF-7 and MDA-MB-231 cells (lane 1 and lane 2, respectively). As a positive control, MCF-7 cells were treated with TNF-α (lane 6). TNF-α, is an activator of caspase-8-dependent apoptotic pathway [Benoit et al., 2004], and it leads to decrease in procaspase-8 levels (lane 6). These results imply that activation of Fas/FasL pathway is responsible for decrease in procaspase-8 levels in the target cells.

Apoptotic Cell Death in Bystander Target Cells

Because our results demonstrated the involvement of FasL/Fas as well as decrease in procaspase-8, we investigated if this would lead to the induction of apoptosis in bystander target cells. To answer this question, we took advantage of measuring cells simultaneously for both phycoerythrin (PE)-conjugated annexin V staining and EGFP positive counts by FACS analysis. The percentage of effector and target cells undergoing apoptosis was determined by their PE-conjugated annexin V staining. As shown in Figure 5B, panel d, when target

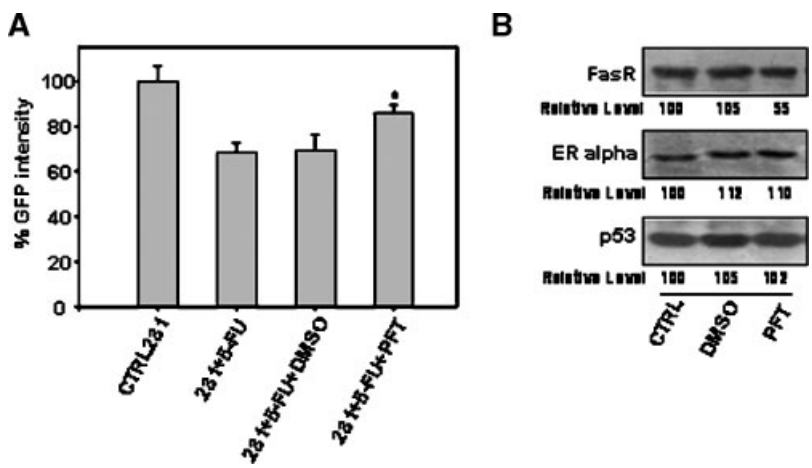

Fig. 4. p53 and Fas receptor are involved in target cell death. **A**: Effect of PFTα, which specifically inhibits wild-type p53 activity, on MCF-EGFP cell survival. Error bars represent the mean ± SD from the results of triplicate experiments (*$P < 0.05$ vs. drug-treated effector cells). The GFP intensity was taken as 100% for cocultures where effector cells were not treated with 5-FU. **B**: A representative western blot showing levels of Fas receptor (FasR) expression on the cell surface of target MCF-EGFP cells when treated with PFTα. Relative levels were measured by densitometry with control normalized to 100 and compared with characteristic estrogen receptor alpha (ERα) levels on the cell membrane.

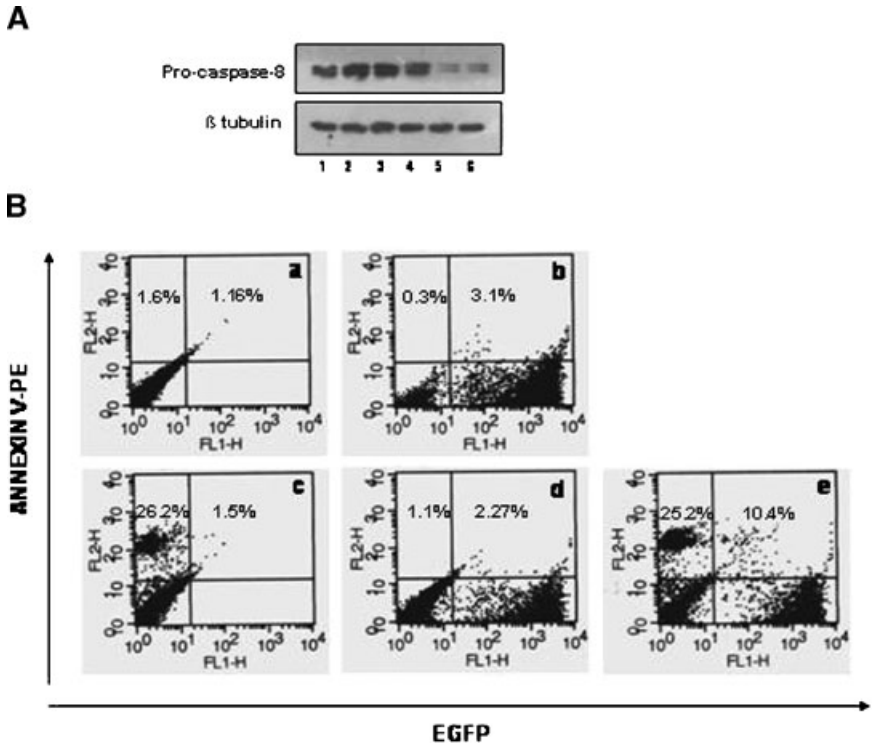

Fig. 5. Caspase-8 activation leads to apoptotic cell death in target cells. **A**: Western blot analysis for total procaspase-8 levels. In untreated MCF-7 cell (**lane 1**); untreated MDA-MB-231 cells (**lane 2**); cocultured MCF-7 with untreated MDA-MB-231 cells (**lane 3**); 5-FU-treated MDA-MB-231 cells (**lane 4**); cocultured MCF-7 cells with 5-FU-treated MDA-MB-231 cells (**lane 5**); and MCF-7 cells alone treated with TNF-α (10 ng/ml), which served as positive control for procaspase-8 cleavage (**lane 6**). **B**: Apoptotic cell death in bystander target MCF-EGFP cells. Histograms for effector and target cell populations alone or in coculture are shown after annexin V-PE staining. Untreated MDA-MB-231 cells (**panel a**); untreated MCF-EGFP cells (**panel b**); 5-FU-treated MDA-MB-231 cells (**panel c**). MCF-EGFP cells cocultured with untreated MDA-MB-231 cells (**panel d**) and MCF-EGFP cells cocultured with 5-FU-treated MDA-MB-231 cells (**panel e**) are shown with annexinV-PE positive counts indicated as percentages of apoptotic cells. Upper right quadrant (panel e) represents proportion of apoptotic bystander MCF-EGFP cells.

MCF-EGFP cells were cocultured with untreated effector MDA-MB-231 cells, barely about 2% of target MCF-EGFP cells were positively stained for annexin V-PE. However, when the target MCF-EGFP cells were cocultured with 5-FU-treated effector MDA-MB-231 cells, about 10% of these GFP positive cells were positive for annexin V-PE (Fig. 5B, panel e). These results clearly indicate induction of apoptosis in bystander target cells. The untreated effector as well as target cell by themselves had a very small basal population of apoptotic cells (Fig. 5B, panels a and b, respectively). In case of effector cells alone exposed to 5-FU, more than 25% cells were apoptotic as analyzed by annexin V-PE positive counts (Fig. 5B, panel c). This was identical to the values obtained when 5-FU-treated effector cells were cocultured with the target cells (Fig. 5B, panel e). The apoptosis of effector cells was not affected when they were cocultured with target cells.

DISCUSSION

Killing of tumor cells by cytotoxic therapies, for example, chemotherapy, radiotherapy, immunotherapy, or suicide gene therapy is predominantly mediated by triggering injury in cancer cells. Cancer chemotherapy suffers major drawback as chemoresistant cells develop within the tumors due to their heterogeneous nature, most importantly in response to therapy under different treatment regimens, and due to inadequate drug delivery methods. Therefore, it is becoming apparent that better understanding of mechanism of drug-induced injury to cancer cells will have profound effect on cancer treatment and management.

Breast cancers are heterogeneous in nature and often progress from a low-invasive estrogen sensitive phenotype to high-invasive, estrogen insensitive phenotype. MCF-7 cell line is prototype for estrogen sensitive breast cancer cells,

representative of relative early stages in breast cancer development. The data presented here demonstrates that 5-FU-treated aggressive breast cancer cells MDA-MB-231, induce bystander killing of MCF-7 breast cancer cells, even though they are not exposed directly to the drug itself. No such bystander cell killing was observed with Carb, another commonly used drug for treating solid tumors like breast cancer. Therefore, 5-FU may potentially be of more therapeutic value than other chemotherapeutic drugs used in the management of aggressive breast cancers because of its ability to initiate bystander cell killing.

It has been demonstrated in earlier studies that following alpha particles irradiation, a larger population of cells were affected than those actually have been hit directly by alpha particles [Nagasawa and Little, 1992; Hall, 2003]. Additionally, there are reports on the enhancement of the Herpes simplex virus thymidine kinase/ganciclovir induced BE and its antitumor efficacy in vivo by pharmacologic manipulation of gap junctions. Furthermore, enhanced cell killing by gene therapy used for prodrug activation also underscored the enhanced therapeutic efficacy of BEs [Touraine et al., 1998; Zhou et al., 2000]. Moreover, since chemotherapeutic drugs can induce apoptosis and upregulate death ligands (TNF, FasL, and TRAIL) or their receptors that may subsequently play a significant role in death signal amplification via BE in a mixed population of cells [Petak and Houghton, 2001]. Finally, the death receptor based amplification of the therapeutic response may be clinically meaningful since it may critically affect the time required for execution of the death program and death receptor based immune clearance of tumor cells [Micheau et al., 1997; Solary et al., 1998; Poulaki et al., 2001]. Taken together, these studies indicate that the bystander phenomenon has great implications in cancer therapies due to the large therapeutic index that can be achieved.

Determining the factors involved in drug response is a new challenge in modern chemotherapy. Therefore, to investigate the molecular pathways that may be contributing to bystander cell death, we exploited the involvement of FasL/Fas and role of tumor suppressor p53. Even though number of investigations have shown induction of apoptosis in a variety of cancer cell types treated with anticancer agents like doxorubicin, cytarabine, etoposide (VP-16), cisplatin, bleomycin, as well as antimetabolites such as methotrexate and 5-FU [Houghton et al., 1997; Micheau et al., 1997; Friesen et al., 1999; Mo and Beck, 1999; Tillman et al., 1999] is mediated via Fas/FasL system, none have shown its involvement in bystander killing of heterogeneous breast cancer cells. Moreover anticancer drugs are known to enhance FasL expression [Micheau et al., 1997]. FasL, which functions as an autocrine/paracrine mediator of apoptosis induced by DNA damaging anticancer chemotherapeutic agents, is a member of the TNF superfamily. It induces apoptosis in susceptible cells upon cross-linking to its own receptor, FAS (Apo-1/CD95).

We observed that upregulation of FasL in MDA-MB-231 exposed to 5-FU induces bystander killing of MCF-7 cells, whereas vice-versa no such effect is observed either in any mixed culture assay or when only single cell type is utilized for the experiment. These results are in agreement with the reports that higher expression of FasL in vivo has been found in tumors with more aggressive clinical behavior as that of MDA-MB-231 cells or tumors with poor histological prognosis, supporting a role for this cytolytic molecule in cancer progression [Gratas et al., 1998; O'Connell et al., 1999]. It has also been reported that MDA-MB-231 cells are resistant to Fas-induced apoptosis, whereas MCF-7 cells are Fas-sensitive [Toillon et al., 2002]. The conditional media obtained from the treated cells as well as the application of gap junction enhancer did not enhance MCF-EGFP cell death. The results suggest the involvement of membrane associated Fas/FasL rather than soluble FasL.

In addition to FasL/Fas pathways, it has been well documented that p53 and factors dependent on p53 potentiate cell death in chemo and radiation therapy of various cancers models [Komarova et al., 1998; Nishizaki et al., 1999]. Especially in the context of p53-mediated gene therapy, adenovirally delivered p53 (AdWTp53) has been evaluated for induction of in vitro as well as in vivo bystander cytotoxicity [Rizk et al., 1999]. Additionally, p53, in addition to its intrinsic antiproliferation activity, can be both, the cause and the target of BE by inducing export of growth suppressive stimuli from damaged cells to neighboring cells. Consistent with this observation, a p53-dependent

accumulation of factors, which causes growth inhibitory effects in a variety of cell lines, were found after gamma irradiation in the media from established and primary cell cultures and in the urine of irradiated mice [Komarova et al., 1998]. Thus in our experiments, the chemotherapy-induced BE was also examined for its dependency on wild-type tumor suppressor p53 protein expressed in the target MCF-EGFP cells [Ozbun and Butel, 1995]. We demonstrate that cell surface expression of Fas receptor was downregulated by inhibition of p53 transcription activity [Bennett et al., 1998]. This resulted in diminished FasL-induced bystander cytotoxicity in these target cells.

Though therapeutic strategies capable of modulating death receptor signaling pathways in combination with anticancer agents is only in its infancy, however, the recent increase of 5-FU-based chemotherapy in breast cancer, gives clinical relevance to our work as it provides experimental basis for understanding and predicting the role of Fas/FasL system in the outcome of fluoropyrimidine-based chemotherapies in the heterogeneous breast tumor. Since little is known about the relationship and dynamics between the levels of soluble and membrane bound Fas and FasL, the balance between all of these apoptotic proteins and/or intracellular defects in downstream signaling may be important for tumor growth, metastasis, and chemotherapy-induced bystander cell death [Mitsiades et al., 1998]. Moreover, an improved understanding of the cellular and molecular mechanisms of bystander phenomenon, together with evidence of their occurrence in vivo, will facilitate better outcome of specific cancer chemotherapy.

ACKNOWLEDGMENTS

We thank Dr. G.C. Mishra, Director, NCCS for being very supportive and giving all the encouragement to carry out this work. R.C. thanks Council for Scientific and Industrial Research for (CSIR) for providing fellowship. We thank Ms. Hemangini for FACS analysis, and all other technical staffs of NCCS are also duly acknowledged.

REFERENCES

Albanese J, Dainiak N. 2000. Ionizing radiation alters Fas antigen ligand at the cell surface and on exfoliated plasma membrane-derived vesicles: Implications for apoptosis and intercellular signaling. Radiat Res 153:49–61.

Ashkenazi A, Dixit VM. 1998. Death receptors: Signaling and modulation. Science 281:1305–1308.

Azzam EI, de Toledo SM, Little JB. 2001. Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha-particle irradiated to nonirradiated cells. Proc Natl Acad Sci USA 98:473–478.

Azzam EI, de Toledo SM, Little JB. 2003. Oxidative metabolism, gap junctions and the ionizing radiation-induced bystander effect. Oncogene 22:7050–7057.

Bennett M, Macdonald K, Chan SW, Luzio JP, Simari R, Weissberg P. 1998. Cell surface trafficking of Fas: A rapid mechanism of p53-mediated apoptosis. Science 282:290–293.

Benoit V, Chariot A, Delacroix L, Deregowski V, Jacobs N, Merville MP, Bours V. 2004. Caspase-8-dependent HER-2 cleavage in response to tumor necrosis factor alpha stimulation is counteracted by nuclear factor kappaB through c-FLIP-L expression. Cancer Res 64:2684–2691.

Cheng TL, Wei SL, Chen BM, Chern JW, Wu MF, Liu PW, Roffler SR. 1999. Bystander killing of tumour cells by antibody-targeted enzymatic activation of a glucuronide prodrug. Br J Cancer 79:1378–1385.

Chipman JK, Mally A, Edwards GO. 2003. Disruption of gap junctions in toxicity and carcinogenicity. Toxicol Sci 71:146–153.

Ciccolini J, Fina F, Bezulier K, Giacometti S, Roussel M, Evrard A, Cuq P, Romain S, Martin PM, Aubert C. 2002. Transmission of apoptosis in human colorectal tumor cells exposed to capecitabine, Xeloda, is mediated via Fas. Mol Cancer Ther 1:923–927.

Djordjevic B. 2000. Bystander effects: A concept in need of clarification. Bioessays 22:286–290.

Friesen C, Fulda S, Debatin KM. 1999. Cytotoxic drugs and the CD95 pathway. Leukemia 13:1854–1858.

Gratas C, Tohma Y, Barnas C, Taniere P, Hainaut P, Ohgaki H. 1998. Up-regulation of Fas (APO-1/CD95) ligand and down-regulation of Fas expression in human esophageal cancer. Cancer Res 58:2057–2062.

Grignet-Debrus C, Cool V, Baudson N, Velu T, Calberg-Bacq CM. 2000. The role of cellular- and prodrug-associated factors in the bystander effect induced by the Varicella zoster and Herpes simplex viral thymidine kinases in suicide gene therapy. Cancer Gene Ther 7:1456–1468.

Guchelaar HJ, Vermes A, Vermes I, Haanen C. 1997. Apoptosis: Molecular mechanisms and implications for cancer chemotherapy. Pharm World Sci 19:119–125.

Hall EJ. 2003. The bystander effect. Health Phys 85:31–35.

Hofmann A, Blau HM. 1997. Death of solid tumor cells induced by Fas ligand expressing primary myoblasts. Somat Cell Mol Genet 23:249–257.

Houghton JA, Harwood FG, Tillman DM. 1997. Thymineless death in colon carcinoma cells is mediated via fas signaling. Proc Natl Acad Sci USA 94:8144–8149.

Hyer ML, Sudarshan S, Schwartz DA, Hannun Y, Dong JY, Norris JS. 2003. Quantification and characterization of the bystander effect in prostate cancer cells following adenovirus-mediated FasL expression. Cancer Gene Ther 10:330–339.

Kagawa S, He C, Gu J, Koch P, Rha SJ, Roth JA, Curley SA, Stephens LC, Fang B. 2001. Antitumor activity and

bystander effects of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene. Cancer Res 61:3330–3338.

Komarova EA, Diatchenko L, Rokhlin OW, Hill JE, Wang ZJ, Krivokrysenko VI, Feinstein E, Gudkov AV. 1998. Stress-induced secretion of growth inhibitors: A novel tumor suppressor function of p53. Oncogene 17:1089–1096.

Konemann S, Schuck A, Malath J, Rupek T, Horn K, Baumann M, Vormoor J, Rube C, Willich N. 2000. Cell heterogeneity and subpopulations in solid tumors characterized by simultaneous immunophenotyping and DNA content analysis. Cytometry 41:172–177.

Li Y, Rosen JM. 2005. Stem/progenitor cells in mouse mammary gland development and breast cancer. J Mammary Gland Biol Neoplasia 10:17–24.

Mesnil M, Yamasaki H. 2000. Bystander effect in herpes simplex virus-thymidine kinase/ganciclovir cancer gene therapy: Role of gap-junctional intercellular communication. Cancer Res 60:3989–3999.

Micheau O, Solary E, Hammann A, Martin F, Dimanche-Boitrel MT. 1997. Sensitization of cancer cells treated with cytotoxic drugs to fas-mediated cytotoxicity. J Natl Cancer Inst 89:783–789.

Mitsiades N, Poulaki V, Kotoula V, Leone A, Tsokos M. 1998. Fas ligand is present in tumors of the Ewing's sarcoma family and is cleaved into a soluble form by a metalloproteinase. Am J Pathol 153:1947–1956.

Mo YY, Beck WT. 1999. DNA damage signals induction of fas ligand in tumor cells. Mol Pharmacol 55:216–222.

Mothersill C, Seymour CB. 2004. Radiation-induced bystander effects—Implications for cancer. Nat Rev Cancer 4:158–164.

Muller M, Wilder S, Bannasch D, Israeli D, Lehlbach K, Li-Weber M, Friedman SL, Galle PR, Stremmel W, Oren M, Krammer PH. 1998. p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. J Exp Med 188:2033–2045.

Nagasawa H, Little JB. 1992. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Res 52:6394–6396.

Nakajima K, Yamauchi K, Shigematsu S, Ikeo S, Komatsu M, Aizawa T, Hashizume K. 2000. Selective attenuation of metabolic branch of insulin receptor down-signaling by high glucose in a hepatoma cell line, HepG2 cells. J Biol Chem 275:20880–20886.

Nishizaki M, Fujiwara T, Tanida T, Hizuta A, Nishimori H, Tokino T, Nakamura Y, Bouvet M, Roth JA, Tanaka N. 1999. Recombinant adenovirus expressing wild-type p53 is antiangiogenic: A proposed mechanism for bystander effect. Clin Cancer Res 5:1015–1023.

O'Connell J, Bennett MW, O'Sullivan GC, O'Callaghan J, Collins JK, Shanahan F. 1999. Expression of Fas (CD95/APO-1) ligand by human breast cancers: Significance for tumor immune privilege. Clin Diagn Lab Immunol 6:457–463.

Ozbun MA, Butel JS. 1995. Tumor suppressor p53 mutations and breast cancer: A critical analysis. Adv Cancer Res 66:71–141.

Petak I, Houghton JA. 2001. Shared pathways: Death receptors and cytotoxic drugs in cancer therapy. Pathol Oncol Res 7:95–106.

Poulaki V, Mitsiades CS, Mitsiades N. 2001. The role of Fas and FasL as mediators of anticancer chemotherapy. Drug Resist Updat 4:233–242.

Rizk NP, Chang MY, El Kouri C, Seth P, Kaiser LR, Albelda SM, Amin KM. 1999. The evaluation of adenoviral p53-mediated bystander effect in gene therapy of cancer. Cancer Gene Ther 6:291–301.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME. 1998. Two CD95 (APO-1/Fas) signaling pathways. EMBO J 17:1675–1687.

Schneider P, Holler N, Bodmer JL, Hahne M, Frei K, Fontana A, Tschopp J. 1998. Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. J Exp Med 187:1205–1213.

Scott BR. 2004. A biological-based model that links genomic instability, bystander effects, and adaptive response. Mutat Res 568:129–143.

Shao C, Folkard M, Michael BD, Prise KM. 2005. Bystander signaling between glioma cells and fibroblasts targeted with counted particles. Int J Cancer 116:45–51.

Sharifi-Salamatian V, Pesquet-Popescu B, Simony-Lafontaine J, Rigaut JP. 2004. Index for spatial heterogeneity in breast cancer. J Microsc 216:110–122.

Solary E, Micheau O, Dimanche-Boitrel MT, Martin F. 1998. The Fas/Fas-ligand system: Implications in the antitumor immune response and in the activity of cytotoxic agents. Bull Cancer 85:685–694.

Symmans WF, Liu J, Knowles DM, Inghirami G. 1995. Breast cancer heterogeneity: Evaluation of clonality in primary and metastatic lesions. Hum Pathol 26:210–216.

Tanaka T, Yamasaki H, Mesnil M. 2001. Stimulation of intercellular communication of poor-communicating cells by gap-junction-competent cells enhances the HSV-TK/GCV bystander effect in vitro. Int J Cancer 91:538–542.

Tillman DM, Petak I, Houghton JA. 1999. A Fas-dependent component in 5-fluorouracil/leucovorin-induced cytotoxicity in colon carcinoma cells. Clin Cancer Res 5:425–430.

Toillon RA, Descamps S, Adriaenssens E, Ricort JM, Bernard D, Boilly B, Le B X. 2002. Normal breast epithelial cells induce apoptosis of breast cancer cells via Fas signaling. Exp Cell Res 275:31–43.

Touraine RL, Vahanian N, Ramsey WJ, Blaese RM. 1998. Enhancement of the herpes simplex virus thymidine kinase/ganciclovir bystander effect and its antitumor efficacy in vivo by pharmacologic manipulation of gap junctions. Hum Gene Ther 9:2385–2391.

Van Triest B, Pinedo HM, Giaccone G, Peters GJ. 2000. Downstream molecular determinants of response to 5-fluorouracil and antifolate thymidylate synthase inhibitors. Ann Oncol 11:385–391.

Xu M, Kumar D, Srinivas S, Detolla LJ, Yu SF, Stass SA, Mixson AJ. 1997. Parenteral gene therapy with p53 inhibits human breast tumors in vivo through a bystander mechanism without evidence of toxicity. Hum Gene Ther 8:177–185.

Zhou D, Lu Y, Steiner MS, Dalton JT. 2000. Cytochrome P-450 2C9 sensitizes human prostate tumor cells to cyclophosphamide via a bystander effect. Antimicrob Agents Chemother 44:2659–2663.